Nicotine directly affects milk production in lactating mammary epithelial cells concurrently with inactivation of STAT5 and glucocorticoid receptor *in vitro*

Ken Kobayashi[1*], Yusaku Tsugami[1], Norihiro Suzuki[1], Takahiro Suzuki[1], and Takanori Nishimura[1]

[1] Laboratory of Cell and Tissue Biology, Research Faculty of Agriculture, Hokkaido University, North 9, West 9, 060-8589, Sapporo, Japan

Corresponding author: Ken Kobayashi, Ph.D.

Laboratory of Cell and Tissue Biology, Research Faculty of Agriculture, Hokkaido University, North 9, West 9, Sapporo 060-8589, Japan

Phone: +81-11-706-2540, FAX: +81-11-706-2540

E-mail: kkobaya@anim.agr.hokudai.ac.jp

Abstract

Nicotine from tobacco smoke is absorbed into the bloodstream and transferred into breast milk in breastfeeding mothers. Smoking causes a decrease in breast milk volume, adverse changes to the milk composition, and a shortened lactation period. Breast milk is produced by mammary epithelial cells (MECs) in mammary glands during lactation. However, it remains unclear whether nicotine directly affects milk production in lactating MECs. To address this issue, we prepared a culture model with high milk production ability and less-permeable tight junctions (TJs) by seeding mouse MECs on a cell culture insert. Lactating MECs showed expression of α2, α3, β2, and β4 of nicotinic acetylcholine receptors. The high concentration of nicotine at 10-100 μM inhibited β-casein secretion and caused abnormal localization of TJ proteins. We subsequently investigated whether nicotine at a physiological concentration could affect lactating MECs. Nicotine at 1.0 μM directly inhibited α- and β-casein secretion in lactating MECs concurrently with inactivation of STAT5 and glucocorticoid receptor without affecting the TJ barrier. Nicotine treatment also induced MEC apoptosis concurrently with inactivation of Akt. These results support the adverse effects of nicotine on breastfeeding in smoking mothers.

Keywords: Mammary epithelial cell, nicotine, milk production, tight junction, apoptosis

1. Introduction

Nicotine from tobacco smoke is rapidly absorbed into the bloodstream, and maternal nicotine is transferred from the bloodstream into breast milk (Napierala et al., 2016). The milk/serum concentration ratio for nicotine is, on average, 2.92±1.09 (Luck and Nau, 1985). Therefore, maternal nicotine use leads to significant exposure to suckling infants during lactation (Luck and Nau, 1987; Primo et al., 2013). Nicotine from maternal milk adversely affects suckling infants, causing, for example, emphysema (Maritz, 2002), heart rate variability (Dahlstrom et al., 2008), and histopathological changes in the lung and liver (Ozokutan et al., 2005). Furthermore, smoking induces a decrease in breast milk volume, adverse changes in the milk composition, and a shorter lactation period (Napierala et al., 2016). Victora et al. have suggested that the scaling up of breastfeeding to a near universal level could prevent 823,000 annual deaths in children younger than 5 years and 20,000 annual deaths from breast cancer (Victora et al., 2016). However, it remains unclear how nicotine adversely affects breastfeeding in smoking mothers.

Breast milk is produced by mammary epithelial cells (MECs) in mammary glands during lactation (Anderson et al., 2007). Lactating MECs synthesize major milk components and secrete them into the alveolar rumen. MECs also form less-permeable tight junctions (TJs) consisting of claudin-3 (CLDN3) and occludin (OCLN) after parturition (Baumgartner et al., 2017). The TJs seal the paracellular pathway between MECs and block leakage of milk and blood components during lactation (Stelwagen and Singh, 2014). Both milk production and TJ formation in MECs are induced after parturition by activation of the STAT5 and glucocorticoid receptor (GR) pathways (Groner, 2002). In contrast, the downregulation of milk production and disruption of TJs occurs through activation of the STAT3 and NFκB signaling pathways in MECs *in vivo* and *in vitro* (Sargeant et al., 2014; Scribner et al., 2011). Thus, milk production and less-permeable TJ formation is dependent on the activation/inactivation of these signaling pathways.

Human and experimental research has clearly shown that nicotine in maternal blood reduces plasma prolactin levels (Andersen et al., 1982; Bahadori et al., 2013; Blake and Sawyer, 1972). Prolactin is a lactogenic hormone that activates STAT5 signaling in mammary epithelial cells (MECs) (Miyoshi et al., 2001). Larger milk yields in women also entail a reduction of somatostatin levels, whereas smoking women show higher levels of this hormone and shorter breast-feeding periods than nonsmokers (Widstrom et al., 1991). These reports suggest that the adverse effects of nicotine on lactating MECs are caused by changing hormone concentrations in the blood plasma. However, MECs express nicotinic acetylcholine receptors (nAChRs) (Kalantari-Dehaghi et al., 2015). In addition, the transfer of nicotine from the bloodstream into breast milk indicates that lactating MECs are exposed to nicotine in smoking mothers (Luck and Nau, 1985). Nicotine is known to influence several signaling pathways including GR, STAT3 and NFκB (Marrero and Bencherif, 2009; Xu et al., 2012), which are involved in milk production and TJs in MECs (Groner, 2002; Sargeant et al., 2014; Scribner et al., 2011). Thus, it is possible that nicotine directly influences lactating MECs.

We have previously reported an *in vitro* model of lactating MECs by cotreatment of prolactin and dexamethasone at 39°C (Kobayashi et al., 2017; Kobayashi et al., 2016; Kobayashi et al., 2018). The model shows active secretion of milk components such as caseins, lactose and triglycerides. It also shows a less-permeable TJ barrier with a continuous network of CLDN3 and OCLN, which seal the paracellular pathway between MECs. In this study, we investigated whether nicotine could directly affect milk production in lactating MECs by using the *in vitro* model, in which MECs were cultured on a cell culture insert. Nicotine was added to the medium in the lower chamber of the cell culture insert to expose the basolateral membranes of MECs to nicotine similarly to nicotine exposure from the bloodstream *in vivo*. The results clearly showed a direct influence of nicotine on milk production by MECs *in vitro*.

2. Materials and Methods

2.1. Animals

Virgin (9–14 weeks) and pregnant ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained under a 12-hour light-dark interval at 22–25°C. The virgin mice were decapitated, and then the fourth mammary glands were collected for isolation of MECs using a cell culture. In addition, the pregnant mice were used for isolation of mRNA of the fourth mammary glands and brain 10 days after parturition (lactation day 10). All experimental procedures in this study were approved by the Animal Resource Committee of Hokkaido University (#14-0005) and conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

2.2. Materials

RPMI-1640 medium, insulin, collagenase, and penicillin-streptomycin solution were obtained from Wako (Osaka, Japan). Cell culture inserts (0.4-μm pore size; BD Biosciences, San Diego, CA) and epidermal growth factor (EGF) were obtained from BD Biosciences (Bedford, MA). The (−) nicotine (99%, liquid), dexamethasone and bovine pituitary extracts containing prolactin were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was from GIBCO-BRL (Grand Island, NY).

The following served as primary antibodies for immunostaining: rabbit polyclonal antibodies against CLDN3 (Thermo Fisher Scientific, Waltham, MA, # 34-1700, 1:300), CLDN4 (Thermo, # 36-4800, 1:200), GM130 (Abcam, Cambridge, UK, # ab52649, 1:500), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, # 9664, 1:400); mouse monoclonal antibodies against OCLN (Thermo, # 33-1500, 1:800); guinea pig polyclonal antibody against adipophilin (Progen,

Heidelberg, Germany, # GP40, 1:600); and goat polyclonal antibody against β-casein (Santa Cruz Biotechnology, Santa Cruz, CA, # M7249, 1:200). The secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody, Alexa Fluor 546-conjugated goat anti-mouse IgG antibody, Alexa Fluor 546-conjugated donkey anti-rabbit IgG antibody, Alexa Fluor 488-conjugated goat anti-guinea pig IgG antibody, and Alexa Fluor 488-conjugated donkey anti-goat IgG antibody) were purchased from Thermo Fisher Scientific.

For western blotting, rabbit polyclonal antibodies against α_{S1}-casein (Santa Cruz Biotechnology, # sc-98699), lactoferrin (Life Laboratory Company, Yamagata, Japan, # LL-A0013), α-lactalbumin (Bethyl Laboratories, Inc. Montgomery, TX, # A10-124-1), CLDN3 (1:1500), CLDN4 (1:1000), OCLN (Thermo, # 71-1500, 1:800), phospho-STAT5 (pSTAT5; Cell Signaling Technology, # 4322, 1:1000), STAT5 (Cell Signaling Technology, # 9363, 1:1000), glucocorticoid receptor (GR; Sigma-Aldrich, # SAB4501309, 1:1000), phospho-STAT3 (pSTAT3; Cell Signaling Technology, # 9145, 1:1000), STAT3 (Santa Cruz Biotechnology, # sc-482, 1:1000), phospho-NFκB p65 (pNFκB; Cell Signaling Technology, # 9145, 1:1000), NFκB p65 (NFκB; Abcam, # ab7970, 1:1000), phospho-mTOR (pmTOR; Cell Signaling Technology, # 5536, 1:1000), mTOR (Cell Signaling Technology, # 2983, 1:1000), phospho-Akt (pAkt; Cell Signaling Technology, # 4060, 1:1000), Akt (Cell Signaling Technology, # 4691, 1:1000), Bax (Biolegend, San Diego, CA, # 625101, 1:1000), Bcl-2 (Biolegend, # 633501, 1:1000), and goat polyclonal antibodies against β-casein (1:1000) were used with mouse monoclonal antibodies against β-actin (1:20,000, cat# MA1-140, Thermo) as an internal control. The secondary horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies for western blotting were purchased from Sigma-Aldrich.

2.3. a cell culture model

In this study, we prepared a culture model of lactating MECs, as previously reported (Kobayashi et al., 2017; Kobayashi et al., 2016; Kobayashi et al., 2018). Briefly, the mammary glands from virgin mice were minced and incubated in RPMI 1640 medium (Sigma-Aldrich) containing 0.75 mg/ml collagenase for 2 hours at 37°C. After centrifugation, the pellet was resuspended in RPMI 1640 medium containing 0.2% trypsin (Thermo Fisher Scientific) for 5 min. After centrifugation, the pellet was resuspended in RPMI 1640 medium containing 50% FBS and then centrifuged at low speed (3 *g*, 5 min). Trypsin treatment and centrifugation with FBS were repeated to isolate MECs without fibroblasts and myoepithelial cells.

MECs were cultured on a cell culture insert in a 24-well cell culture plate in RPMI 1640 medium supplemented with 5% FBS, 5 μg/ml insulin and 10 ng/ml EGF for 6 days. Subsequently, the MECs were cultured in RPMI 1640 medium containing 1% FBS, 10 μg/ml insulin, 10 ng/ml EGF, 0.45 mg/ml bovine pituitary extract and 1 μM dexamethasone at 37°C for 2 days. They were then cultured at 39°C for 3 days to facilitate the induction of milk production ability and less-permeable TJ formation (Kobayashi et al., 2018). Nicotine was added to the medium in the lower chamber at 0.1-33 μM for the last 1 or 3 days.

2.4. Immunofluorescence staining

For immunofluorescence staining, the cultured MECs were fixed in methanol for 10 min at −20°C and then 1% formaldehyde in PBS for 10 min at 4°C followed by treatment with 0.2% Triton X-100 in PBS for 5 min at room temperature. The fixed MECs were incubated with PBS containing 5% bovine serum albumin (BSA; MP Biomedicals, Solon, OH) to block nonspecific interactions, followed by primary antibody diluted in blocking solution overnight at 4°C. They were then washed with PBS containing 0.05% Tween-20 (PBST) and exposed to secondary antibody for 40 min at room temperature in blocking solution. Controls were treated in the same manner, except

for the exclusion of primary antibody. Images of the stained MECs were obtained using a confocal laser-scanning microscope (TCS SP5; Leica, Mannheim, Germany).

2.5. Western blot analysis

The samples of cultured MECs and conditioned media were electrophoresed on 7.2%, 10%, or 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 1 h with PBST containing 4% nonfat dried milk and then incubated for 2 days at 4°C with primary antibodies diluted in PBST containing 2.5% BSA. For α-, β-casein, lactoferrin, and adipophilin detection, PBST containing 2% BSA was used as the blocking solution. PBST containing 3% fish gelatin was used for blocking and primary antibody dilution for α-lactalbumin detection. Subsequently, the membranes were washed in PBST and incubated for 40 min at room temperature with the appropriate secondary horseradish peroxidase-conjugated antibody diluted in each blocking solution. Immunoreactive bands were detected using Luminata Forte Western HRP substrate (Merck Millipore, Darmstadt, Germany, # WBLUF0500). Images of the protein bands were obtained with a Bio-Rad ChemiDoc™ EQ densitometer and Bio-Rad Quantity One® software (Bio-Rad Laboratories, Hercules, CA) with quantification of the bands by densitometry. Beta-actin was used as an internal control.

2.6. Reverse transcription (RT)-PCR

Total RNA was extracted from cultured MECs, brains and mammary glands of the mice at lactation day 10 using ISOGEN II (Wako). RT was performed using ReverTraAce qPCR RT Master Mix (Toyobo, Osaka, Japan). PCR was conducted on a Life Touch thermal cycler (Life Eco, Bioer Technology, Hangzhou, China) with Quick Taq HS DyeMix (Toyobo) using the following cycling conditions: 94°C for 2 min followed by 40 cycles at 94°C for 30 sec, 58°C for

30 sec, and 68°C for 50 sec. The primer information is provided in Table 1.

2.7. Measurement of TJ permeability

To evaluate the barrier function of TJs, the transepithelial resistance (TER) of the MEC layer cultured on a cell culture insert in a 24-well plate (0.4-μm pore size; BD Biosciences) was measured. The electrodes of a Millicel-ERS system (Millipore) were placed in the upper and lower chambers, and the TER of the MEC layer was measured.

2.8. Statistical analysis

The data are expressed as the mean (standard error of the mean [SEM]). Significance values were calculated using the Bonferroni-corrected two-tailed Student's t-test following one-way analysis of variance (ANOVA). Differences were considered significant at p-values <0.05. All experiments subjected to statistical analysis were performed a minimum of three times using mammary glands originating from different mice.

3. Results

3.1. A culture model of lactating MECs expressing nAChRs

To prepare a lactating MEC culture model, MECs were seeded on a cell culture insert in growth medium (Fig. 1A). After reaching 100% confluency (Fig. 1B), the MECs were cultured in differentiation medium containing prolactin and dexamethasone for 3 days. The milk production ability and TJ formation of MECs was confirmed by immunostaining. Beta-casein, which is a representative major milk protein (Miller et al., 1990), was localized mainly in the Golgi apparatus, as represented by GM130 localization (Fig. 1C). Adipophilin, which is a major protein coating cellular lipid droplet (Anderson et al., 2007), was localized on the surface of lipid droplets in the

MECs (Fig. 1D). The TJ protein CLDN3 was localized along the apical-most regions of the MECs, as represented by the localization of OCLN (Fig. 1E) (Baumgartner et al., 2017).

MECs in the culture model showed expression of *α2*, *α3*, *β2*, and *β4* of the nAChR subtypes by RT-PCR (Fig. 1F). In lactating mammary glands, *α2*, *α3, α4, α6, α7, β2,* and *β4* were detected. The brain showed expression of all the nAChR subtypes examined in this study except *Csn2* of a milk-specific protein. These results suggest that the culture model of lactating MECs is suitable for investigating the direct influence of nicotine on milk production and TJs of MECs. In this study, nicotine was added to the medium in the lower chamber (Fig. 1A).

3.2. Influence of a high concentration of nicotine in β-casein production and TJ protein expression

To confirm whether lactating MECs are nicotine sensitive, MECs were exposed to nicotine for 3 days at 10-100 μM, which is a higher concentration than that detected in the breast milk of smoking mothers (Napierala et al., 2016). The band images obtained by western blotting and the densitometry analysis revealed a decrease in secreted β-casein into the medium in MECs treated with nicotine (Fig. 2A, B). Nicotine treatment at 33 μM also led to an increase in CLDN4 and decrease in intracellular β-casein. Nicotine treatment did not significantly change the amount of CLDN3 at any concentration.

Immunostaining images of MECs treated with 33 μM nicotine treatment for 3 days showed a weak fluorescence intensity in β-casein-positive cells without obvious differences in the localization of GM130, compared to the control (Fig. 2C). CLDN3 was observed in TJ regions with OCLN in MECs regardless of nicotine treatment. The additional localization of CLDN3 was observed in the cytoplasm of nicotine-treated MECs. CLDN4 was clearly localized in the plasma membrane and cytoplasm of MECs treated with nicotine at 33 μM, suggesting that lactating MECs are nicotine sensitive.

3.3. Influence of a physiological concentration of nicotine on milk protein production

Subsequently, we investigated whether nicotine could affect lactating MECs at 0.1 and 1.0 μM of the physiological concentration detected in the maternal milk of the smoking mother (Napierala et al., 2016). The amount of intracellular α- and β-casein was minimally changed by nicotine treatment at 0.1 or 1.0 μM (Fig. 3A, B). The amount of α- and β-casein secreted into the medium was significantly decreased in MECs treated with 1.0 μM nicotine. Nicotine treatment at 0.1 or 1.0 μM did not influence the intracellular and secreted lactoferrin (Fig. 3A, C). Adipophilin, a protein that coats lipid droplets, showed a significant decrease upon treatment with 1.0 μM nicotine (Fig. 3A, D). Alpha-lactalbumin, which is indispensable for lactose synthesis (Ramakrishnan and Qasba, 2001), showed a negligible change in MECs treated with 0.1 or 1.0 μM nicotine.

3.4. Influence of a physiological concentration of nicotine on TJs

The expression patterns of TJ proteins in MECs treated with a physiological concentration of nicotine were investigated by western blotting and immunostaining. Western blot analysis revealed a significant decrease in CLDN4 following 1.0 μM nicotine treatment (Fig. 4A, B). The amount of CLDN3 and OCLN did not significantly change in the presence of 0.1 or 1.0 μM nicotine. In addition, CLDN3 was localized in TJ regions with OCLN, without a detectable difference between MECs that were untreated versus treated with 1.0 μM nicotine (Fig. 4C). The barrier function in the MEC layer was evaluated by measuring TER. The MEC layer treated with 1.0 μM nicotine showed a similar TER to the control (Fig. 4D).

3.5. Influence of a physiological concentration of nicotine on milk production-related signaling

pathways

Coactivation of the STAT5 and GR pathways induces milk production in MECs *in vivo* and *in vitro* (Groner, 2002; Kobayashi et al., 2017). In contrast, activation of the STAT3 and NFκB pathways has been observed in mastitis or after weaning with MECs with low or no milk production ability (Dang et al., 2015; Kobayashi et al., 2013). Therefore, we investigated the activation state of these signaling pathways in MECs treated with a physiological concentration of nicotine.

The amounts of pSTAT5 and total STAT5 were examined by western blotting. MECs showed significantly decreased levels of pSTAT5 in response to nicotine treatment in a dose-dependent manner without significant decreases in total STAT5 (Fig. 5A, B). The amount of GR was significantly decreased in MECs treated with 1.0 μM nicotine. In contrast, treatment with 0.1 or 1.0 μM nicotine had a negligible effect on the amount of pSTAT3, total STAT3, pNFκB, or total NFκB in MECs (Fig. 5C, D).

3.6. Influence of a physiological concentration of nicotine on cell survival-related factors.

Nicotine exposure promotes apoptosis resistance in breast cancer cells (Guha et al., 2014). However, it remains unclear whether nicotine exposure affects cell survival or cell death in lactating MECs. Thus, we investigated the cell survival-related factors Akt, mTOR, Bax, Bcl-2, and cleaved caspase-3 (Merto et al., 1997; Shaik et al., 2017).

The amounts of phosphorylated and total mTOR showed minimal changes upon nicotine treatment at 0.1 or 1.0 μM for 24 h (Fig. 6A, B). Phosphorylated-Akt significantly decreased following treatment with 1.0 μM nicotine, although the amount of total Akt did not change. The apoptosis regulator Bax showed a significant decrease in MECs treated with nicotine at 0.1 μM but not 1.0 μM. The anti-apoptotic protein Bcl-2 increased in MECs in response to 1.0 μM

nicotine treatment (Fig. 6A, C). Immunostaining images of the apoptosis marker cleaved caspase-3 revealed apoptotic cells in the MEC layer treated with 1.0 μM, although cleaved caspase-3-positive cells were rarely observed in the MEC layer in the absence of nicotine treatment (Fig. 6D).

4. Discussion

Smoking causes a decline in breast milk volume, adverse alterations to the milk composition, and a shorter lactation period in breastfeeding mothers (Napierala et al., 2016). During lactation, MECs are exposed to a high concentration of nicotine because the breast milk contains more nicotine than the maternal blood plasma (Luck and Nau, 1985). In this study, we investigated the direct influences of nicotine on lactating MECs by using a culture model in which MECs produce milk components and form less-permeable TJs (Kobayashi et al., 2018; Tsugami et al., 2017). MECs also express 4 kinds of nAchR subtypes. In fact, a high concentration of nicotine, 10-100 μM, adversely affected the secretion of β-casein and the expression patterns of CLDNs, suggesting that lactating MECs are nicotine sensitive. The nicotine concentration in the breast milk of the smoking mother ranges from approximately 0.1-1.0 μM (Napierala et al., 2016). In this study, a physiological concentration of nicotine also decreased α- and β-casein secretion concurrently with a decline in adipophilin, a major protein coating cellular lipid droplets (Anderson et al., 2007). Furthermore, nicotine treatment led to the inactivation of STAT5 and GR, which are transcription factors that induce milk production ability in MECs (Groner, 2002; Kobayashi et al., 2017). However, treatment with nicotine at a physiological concentration had minimal effects on the TJ barrier in the MEC layer, excluding a decrease in CLDN4, which is normally downregulated in lactating MECs after parturition and during lactation (Kobayashi and Kumura, 2011). These findings suggest that nicotine directly and adversely affects milk

production by lactating MECs via nAchRs without weakening the TJ barrier.

In the present study, nicotine treatment induced apoptosis in lactating MECs concurrently with inactivation of STAT5 and Akt. Activation of Stat5 promotes the survival of MECs after weaning (Creamer et al., 2010). Activation of Akt in MECs also induces cell survival and delays involution of the mammary gland after weaning (Hutchinson et al., 2001). Nicotine causes a decline in breast milk volume and a shorter lactation period in breastfeeding and smoking mothers (Napierala et al., 2016). The adverse effects of nicotine on breastfeeding of smoking mothers may be caused by apoptosis of lactating MECs via inactivation of the STAT5 and Akt pathways. Conversely, nicotine is well known to promote cell proliferation and cell survival in tumor cells (Dinicola et al., 2013; Wang et al., 2014). Nicotine upregulates anti-apoptotic protein Bcl-2 via α4β2 nAChR (Kihara et al., 1998). In addition, Bax is induced at the onset of apoptosis in involuting mammary epithelial cells (Heermeier et al., 1996). However, nicotine treatment led to an increase in Bcl-2 and a decrease in Bax in MECs, even though apoptosis was induced in MECs in this study. Further investigation is required to unravel the contradiction between cell survival and apoptosis in lactating MECs.

Lactating MECs showed expression of the α2, α3, β2, and β4 subtypes of nAchR. In addition. nicotine treatment induced inactivation of STAT5, GR, and Akt in lactating MECs. In the brain, α7 homometric and α4β2 heterometric nAChRs are the two major subtypes (Gotti et al., 2007). In human placenta, expression of the α2, α3, α4, α9, β2 and β4 subunits has been reported (Machaalani et al., 2014). Each nAChR has distinct functions via activating distinct signaling pathways. For example, α7 nAChR regulates the STAT3, NF-κB, and Akt pathways (Wazea et al., 2018). α4β2 nAChR functions in neuroprotection through the PI3K/Akt, STAT3/JAK2, and MEK/ERK pathways (Buckingham et al., 2009). Crosstalk between these signaling pathways has been reported (Bishop et al., 2014). Crosstalk between the STAT5 and PI3K/Akt pathways also

occurs in normal and transformed MECs (Radler et al., 2017). Activation of the JAK2/STAT5 pathways by binding of prolactin to prolactin receptor is a main signaling event leading to the induction of milk production in MECs (Watson and Burdon, 1996). These findings suggest that nicotine downregulates milk production ability in MECs through inactivation of the JAK2/STAT5 pathways caused by crosstalk between multiple signaling pathways such as Akt. In addition, nicotine-exposed offspring have been reported to exhibit reduced expression of GR in the lung (Suter et al., 2015). Further investigation is required to elucidate the nicotine signaling pathway in lactating MECs.

In this study, we revealed that lactating MECs expressed the α2, α3, β2, and β4 subtypes of nAchR. Nicotine directly and adversely affected milk production in lactating MECs concurrently with inactivation of STAT5 and GR. In addition, nicotine treatment induced apoptosis in MECs. These results support the adverse effects of nicotine on breastfeeding in smoking mothers. However, the main nicotine receptor in lactating MECs and how nicotine causes apoptosis remain unclear. The experiments using several antagonists of nAChR may reveal the main nicotine receptor. Thus, further investigations will be indispensable for breasting mothers.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1

A culture model of lactating MECs expressing nicotine receptor

(A) MECs were cultured on the cell culture insert to prepare a culture model. (B) Phase contrast image of MECs after reaching 100% confluency. (C-E) Immunostaining images of β-casein (green; C) with GM130 (red), adipophilin (green; D), and CLDN3 (green; E) with OCLN (red) in MECs cultured in differentiation medium for 4 days. Blue indicates nuclei stained with DAPI. Scale bars are 10 μm. (F) Band images show the mRNA expression of the nicotinic acetylcholine receptor (nAChR) subtypes (*α2*, *α3*, *α4*, *α5*, *α6*, *α7*, *α9*, *β2*, *β3*, *β4*), β-casein (*Csn2*), and *Gapdh* by RT-PCR in brains and mammary glands dissected from lactating mice at 10 days after parturition, and MECs cultured in differentiation medium for 4 days.

Fig. 2

Influence of a high concentration of nicotine on the expression of β-casein and TJ proteins in MECs

MECs were cultured in differentiation medium containing 10-100 μM nicotine for 3 days. The results of western blotting (A) and densitometry analysis (B) of intracellular and secreted β-casein, CLDN3, and CLDN4 using β-actin as an internal control; n=6. The data are presented as the mean (SEM). *$p<0.05$ versus control. (C) Immunostaining images of β-casein (green), GM130 (red), CLDN3 (green), CLDN4 (green), and OCLN (red) in MECs cultured in differentiation medium containing 33 μM nicotine for 3 days. Scale bars are 10 μm.

Fig. 3

Influence of a physiological concentration of nicotine on milk protein production in MECs

MECs were cultured in differentiation medium containing 0.1 or 1.0 μM nicotine for 24 h. The results of western blotting (A) and densitometry analysis (B-D) of intracellular and secreted α- and β-casein (B), lactoferrin (C), intracellular α-lactalbumin, and adipophilin using β-actin as an internal control; n=8. The data are presented as the mean (SEM). *$p<0.05$ versus control.

Fig. 4

Influence of a physiological concentration of nicotine on TJs in MECs

MECs were cultured in differentiation medium containing 0.1 or 1.0 μM nicotine for 24 h. The results of western blotting (A) and densitometry analysis (B) of CLDN3, CLDN4, and OCLN; n=8. Beta-actin was used as an internal control. The image of the β-actin band is shown in Fig. 3A. The data are presented as the mean (SEM). *$p<0.05$ versus control. (C) Immunostaining images of CLDN3 (green) with OCLN (red) in MECs cultured in differentiation medium containing 1.0 μM nicotine for 24 h. (D) Transepithelial resistance (TER) in MECs treated with 1.0 μM nicotine for 24 h; n=6.

Fig. 5

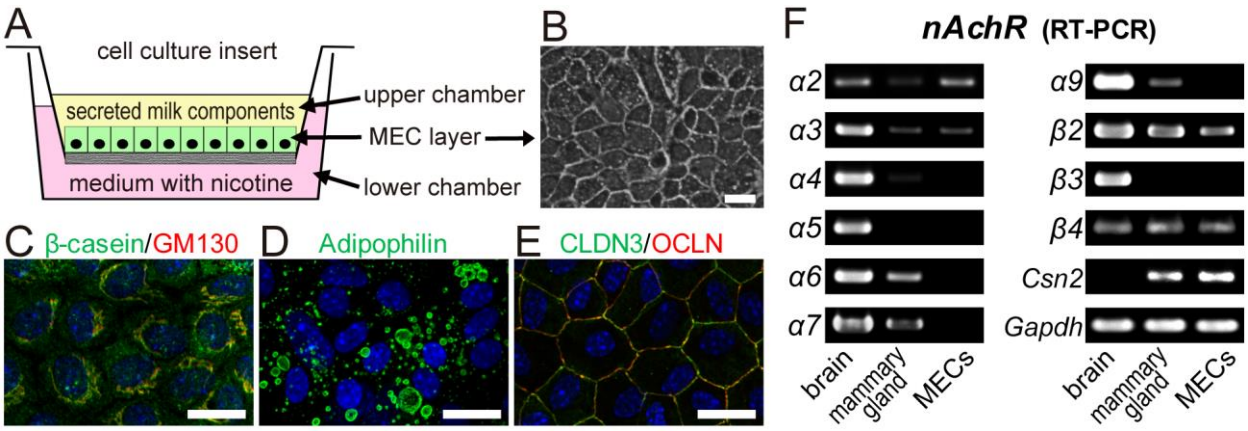
Influence of nicotine at a physiological concentration on signal pathways involving milk production in MECs

MECs were cultured in differentiation medium containing 0.1 or 1.0 μM nicotine for 24 h. The results of western blotting (A, C) and densitometry analysis (B, D) of phosphorylated STAT5 (pSTAT5), STAT5, GR, pSTAT3, STAT3, NFκB, and pNFκB; n=8. Beta-actin was used as an internal control. The image of the β-actin band is shown in Fig. 3A. The data are presented as the mean (SEM). *$p<0.05$, **$p<0.01$ versus control.

Fig. 6

Influence of a physiological concentration of nicotine on cell survival-related factors in MECs

MECs were cultured in differentiation medium containing 0.1 or 1.0 μM nicotine for 24 h. The results of western blotting (A) and densitometry analysis (B, C) of phosphorylated mTOR (pmTOR), mTOR, pAkt, Akt, Bax, and Bcl-2; n=8. Beta-actin was used as an internal control. The data are presented as the mean (SEM). *$p<0.05$ versus control. (D) Immunostaining images of cleaved caspase-3 (green) in MECs cultured in differentiation medium containing 1.0 μM nicotine for 1 day. Blue indicates nuclei stained with DAPI. Scale bars are 20 μm.



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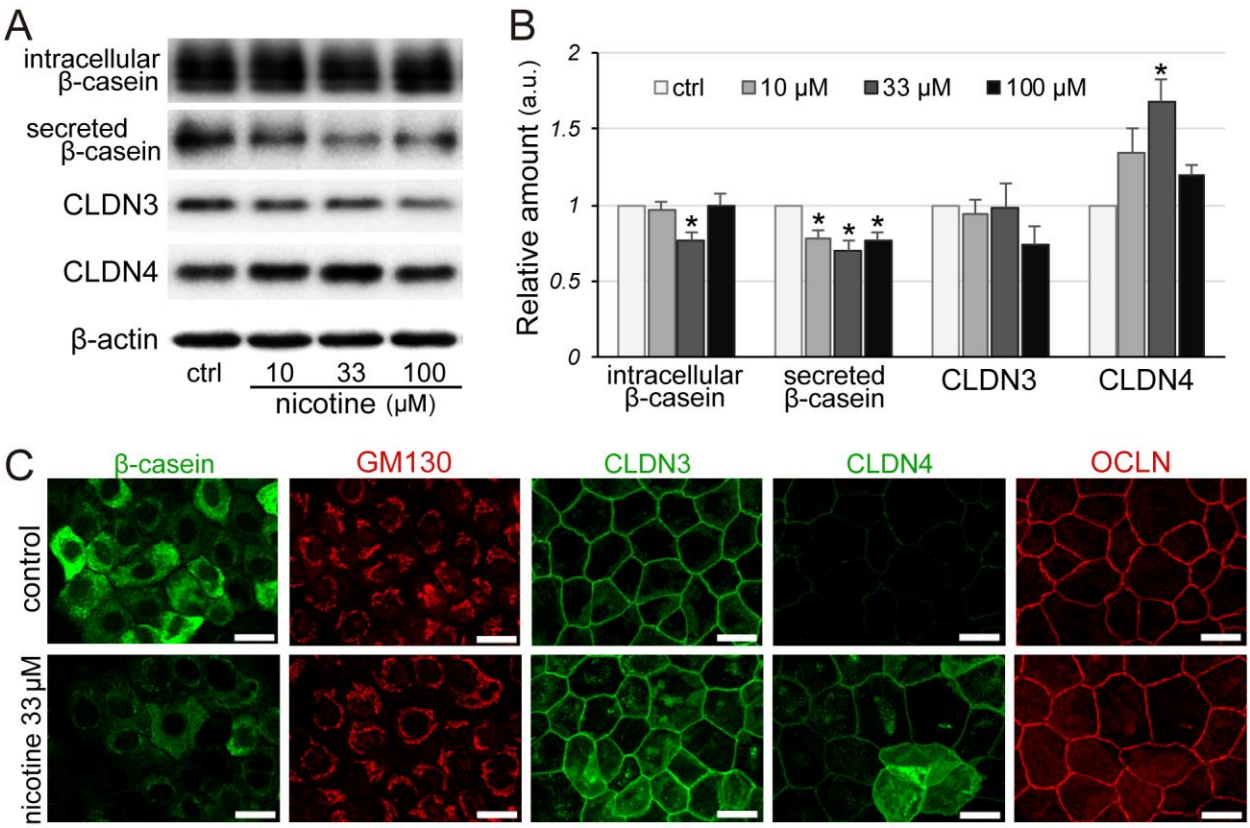

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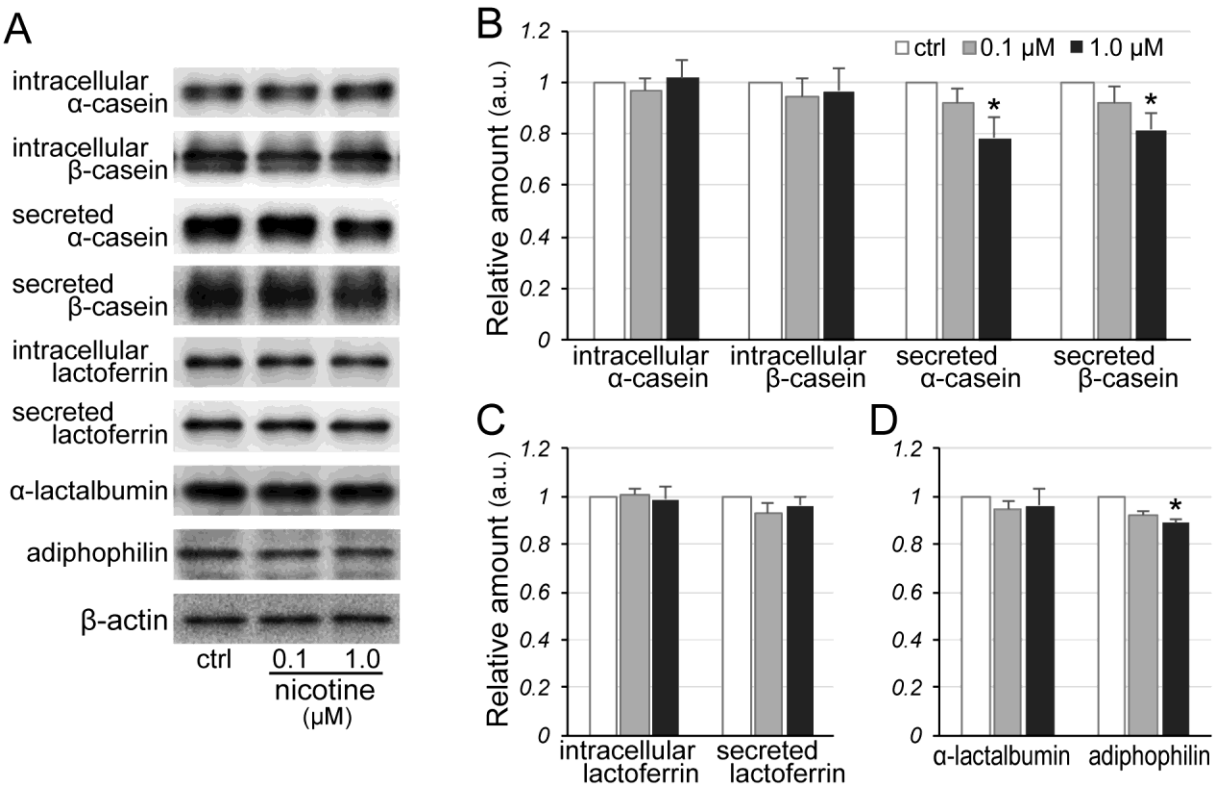

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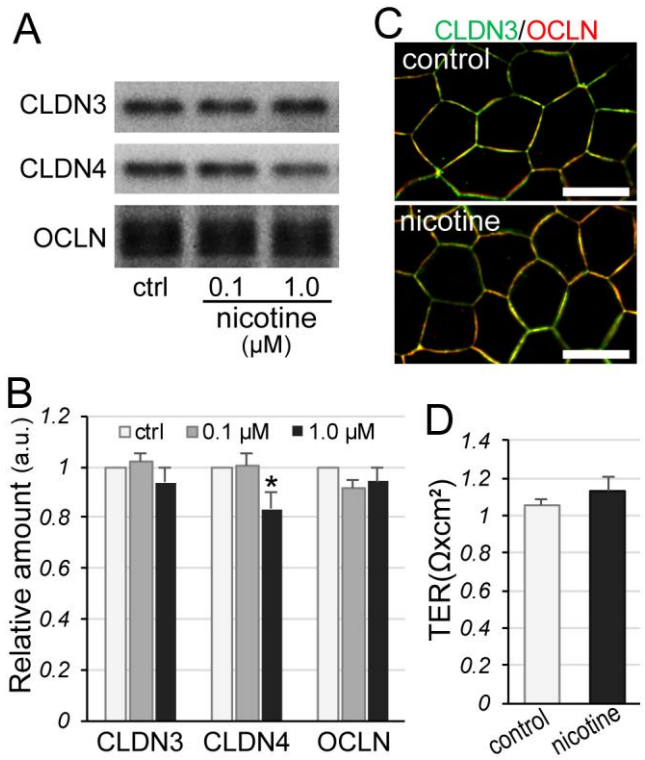

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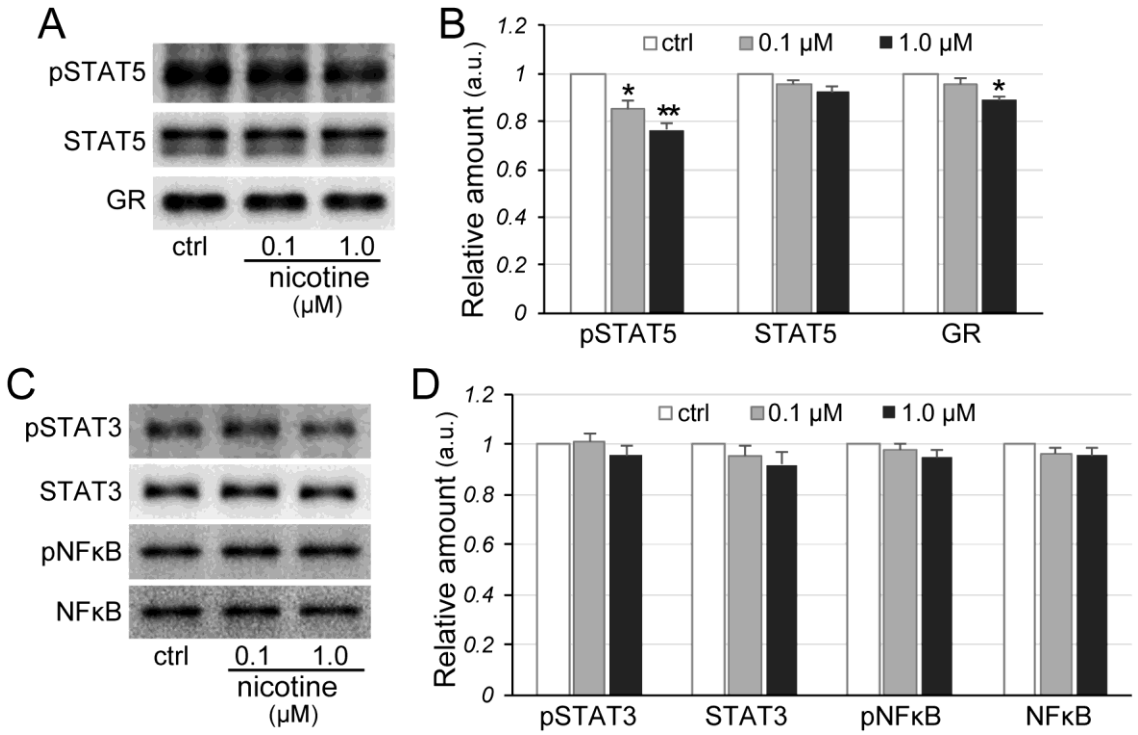

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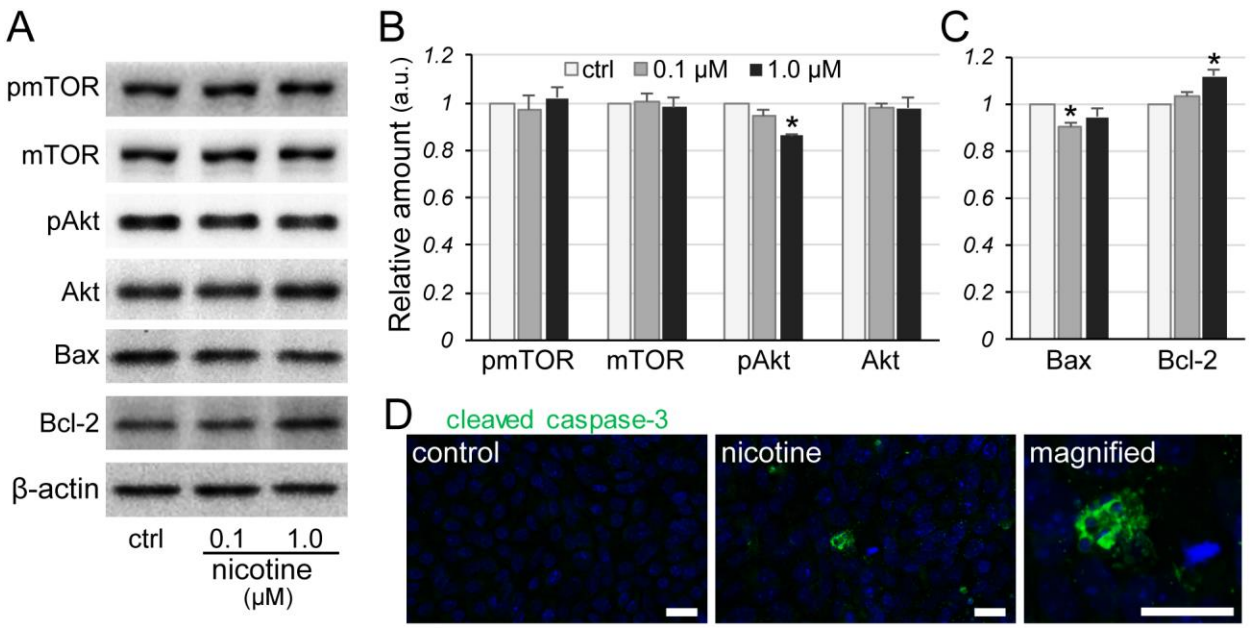

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Table 1
Primer sequences for RT-PCR

Gene	Accession number	Primers		product size
		Forward	Reverse	
nAChR-α2	NM_144803.2	TCTACCCCGACGTCACCTAC	ACACCCATGGAAGAGTCTGG	571
nAChR-α3	NM_145129.3	TGGGGATTTCCAAGTGGA	CATGACCCTGGGGAGAAGGT	679
nAChR-α4	NM_015730.5	CAATGTACACCACCGCTCAC	TGGTCTGACACTGGAAGCTG	594
nAChR-α5	NM_176844.4	CCTCTGCTGCAAAACATGAA	CGCTCATGATTTCCCATTCT	546
nAChR-α6	NM_021369.2	TCCCTGAAGTTTGGTTCCTG	CTCCTGCCTCCTTTGTCTTG	583
nAChR-α7	NM_007390.3	GCACCTCATGCATGGTACAC	CTATCGGGTGAGCCCATGAC	605
nAChR-α9	NM_001081104.1	CCTTGCGTCCTCATATCGTT	CCCTGGAAGTTTGCCATAAA	399
nAChR-β2	NM_009602.4	GGGAAGATTATCGCCTCACA	GCCAGCAGCACAGAAATACA	565
nAChR-β3	NM_173212.4	GCTCAGTGGCTGAACATGAA	TCTTCTGTTGCCCTTCATCC	575
nAChR-β4	NM_148944.4	TCTGGTTGCCTGACATCGTG	CACACAGTGGTGACGATGGA	616
Csn2	NM_009972	CTTCAGAAGGTGAATCTCATGGG	CAGATTAGCAAGACTGGCAAG	330
Gapdh	NM_008084	GAGCGAGACCCCACTAACATC	GCGGAGATGATGACCCTTTT	144